PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Docket No: A8564

Zeev ALTBOUM, et al.

Appln. No.: 09/839,894

Group Art Unit: 1648

Confirmation No.: 4293

Examiner: Lucas, Z.

Filed: April 20, 2001

For: ISOLATION AND CHARACTERIZATION OF THE CSA OPERON (ETEC-CS4 PILI) AND METHODS OF USING SAME

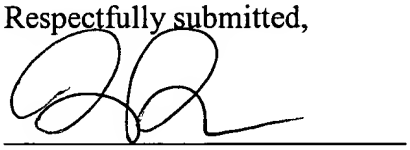
SUBMISSION OF EXECUTED DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith is a copy of an executed Declaration Under 37 C.F.R. §1.132 signed by Myron M. LEVINE and Eileen M. BARRY.

Respectfully submitted,



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Date: April 20, 2004

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DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, **Eileen M. Barry, Ph.D.**, hereby declare and state:

THAT I am a citizen of the United States of America;

THAT I have received the degree of Ph.D. in 1991 from Virginia Commonwealth University;

THAT I have been employed by University of Maryland, Baltimore, since 1992, where I hold a position as Assistant Professor, with responsibility for directing the Shigella Vaccine Unit;

I am familiar with U.S. patent application number 09/839,894 of which I am a listed inventor, and with the Office Action dated October 21, 2003 in said application.

I, **Myron M. Levine, Ph.D.**, hereby declare and state:

THAT I am a citizen of the United States of America;

THAT I have received the degree of M.D. in 1967 from the Medical College of Virginia;

THAT I have been employed by University of Maryland, Baltimore, since September 1, 1973, where I hold a position as Professor, with responsibility for overseeing the Center for Vaccine Development (as Director), the Division of Geographic Medicine in the Department of Medicine, and the Division of Infectious Diseases and Tropical Pediatrics in the Department of Pediatrics (as Division Head);

I am familiar with U.S. patent application number 09/839,894 of which I am a listed inventor, and with the Office Action dated October 21, 2003 in said application.

The following statements are submitted in order to explain the interactions that occur between polypeptides encoded by the *csa* operon of Enterotoxigenic *Escherichia coli* (ETEC), the subject matter of the above-identified patent application.

I. Background

ETEC is a major cause of diarrhea in humans (Black et al., *Lancet*, I: 141-143 (1981); Levine, *J. Infect. Dis.*, 155:377-389 (1987); Qadri, et al., *J. Clin. Microbiol.*, 38:27-31 (2000)). ETEC strains colonize the small bowel lumen by means of surface fimbriae called colonization factor antigens (CFA), and coli surface antigens (CS), and cause diarrhea through the action of heat labile (LT) and/or heat stable (ST) enterotoxins. ETEC fimbriae are proteinaceous filaments exhibiting different morphologies such as rigid rod-like shapes of 2-7 nm in diameter, thin flexible wiry fibrillae, or bundle-forming and non-fimbrial structures (Gaastra et al., *Trends. Microbiol.*, 4:444-452 (1996)).

Human ETEC strains display a variety of over 20 serologically distinct fimbriae on their cell surfaces. The most common human ETEC strains express CFA/I, CFA/II and CFA/IV (Levine, et al., "Fimbrial vaccines," *In* P. Klemm (ed.), Fimbriae: adhesion, biogenics, genetics

and vaccines, Boca Raton: CRC Press, 1994; McConnell, et al., *Epidemiol. Infect.*, 106: 477-484 (1991)). CFA/I produces a single type of fimbriae, while CFA/II and CFA/IV strains produce several types of coli surface antigens. CFA/II strains express CS3, either alone or in conjunction with CS1 or CS2 antigens; and CFA/IV strains (originally called PCF8775) express CS6, either alone or together with CS4 or CS5 fimbriae. (McConnell, et al., *Infect. Immun.*, 56:1974-1980 (1988); McConnell, et al., *FEMS Microbiol. Lett.*, 52:105-108 (1989); Svennerholm, et al., *Infect. Immun.*, 56:523-528 (1988); Thomas, et al., *J. Gen. Microbiol.*, 131:2319-2326 (1985)).

The CS4 antigen is rigid, 7 nm in diameter, and is composed of subunits with a molecular mass of 17.0 kDa. (Knutton, et al., *Infect. Immun.*, 57:3364-3371 (1989); McConnell, et al., *Infect. Immun.*, 56:1974-1980 (1988); Wolf, et al., *Infect. Immun.*, 57:164-173 (1989)). Until the work described in the present application, the complete operon encoding the CS4 antigen had not been cloned or sequenced.

The genes that are required for the expression of functional fimbriae are characteristically linked in gene clusters (Sakellaris and Scott, *Mol. Microbiol.*, 30:681-687 (1998)), and consist of the structural genes, assembly cassette genes and regulatory genes. As now described in detail in the present application, the *csa* operon has been identified and found to encode the elements required for synthesis of the ETEC CS4 fimbriae. This operon was cloned from ETEC strain E11881A, a CS4 producing strain. A nucleic acid fragment comprising 7239 base pairs was sequenced (in both directions). The results indicate that the *csa* operon is located on a nucleic acid fragment 6095 base pairs in length. (Accession No. AF296132).

II. Csa Operon

An analysis of the *csa* operon revealed that it encodes five proteins. By homology with other fimbriae consisting of similar proteins, the CS4 proteins are hypothesized to have the following functions. The proteins are the fimbrial subunit structural protein (CsaB), the tip-associated protein (CsaE), a chaperon-like protein (CsaA), an usher-like protein (CsaC), and a truncated regulatory protein (CsaD).

The CsaB protein consists of 167 amino acids, (23 of which comprise a signal peptide), producing an ~17 kDa peptide. The amino acid sequence of the CsaB protein shares homology with other ETEC fimbrial proteins. For example, CsaB is 67.1% identical to the CfaB protein of CFA/I (accession #M55661), 62.9% identical to CsuA1, the structural subunit of CS14 (Accession #X97491), 58.3% identical to the CS1 structural protein, CooA (Accession #M58550), 51.5% identical to the CS2 structural protein, CotA (Accession #Z47800), 52.7% identical to the CS19 structural protein, CsdA (Accession #X97494) and 57.6% identical to the CS17 structural protein, CsbA (Accession #AY216491).

The CsaE protein is believed to be a tip-associated protein based on its homology to known tip proteins of other ETEC fimbriae. It is a protein of 361 amino acids, 23 amino acids of which are cleaved to produce a globular ~40 kDa protein. The amino acid sequence of the CsaE protein shares homology to CFA/I, CS1 and CS2 fimbrial tip proteins, with identities of 81.1% to CfaE, 53.2% to CooD, and 48.4% to CotD.

The fimbrial assembly proteins CsaA (chaperone protein) and CsaC (usher protein) share homology to similar proteins from CFA/I, CS1 and CS2 pili, 93.3%, 60.2% and 50.8% with regard to CsaA, and 94.6%, 64.7% and 56.2% with regard to CsaC, respectively.

CsaD is believed to be a truncated regulatory protein based on its homology to other known ETEC regulatory proteins.

III. Mechanism of Assembly

Based on common mechanisms found among fimbriae of different ETEC strains (reviewed in Sakellaris et al., *Molecular Micro.* 30:681-687 (1998)), assembly of the fimbriae is thought to begin with binding of the chaperone protein to fimbrial subunit structural proteins in the periplasmic space, preventing premature folding and degradation. The fimbria is composed of repeating subunits of the structural protein, assembled through the actions of the chaperone protein. Addition of the minor pilin tip protein, which is associated with the fimbrial tip, is also added to the growing fimbriae in the periplasmic space though the action of the chaperone-like protein. Upon completion of the fimbria, it is transported to the surface of the bacteria through the actions of the usher-like protein, which is an outer membrane protein that serves as a pore for the transport and assembly of the fimbriae.

Thus, in the case of the CS4 antigen, the CsaB protein is the subunit that forms the primary structure of the antigen. The CsaE protein is the tip-associated protein that is added to the end of the fimbriae (comprised primarily of repeating CsaB proteins). CS4 antigen assembly takes place through the actions of the CsaA protein, which is the chaperone protein required for assembly of the CS4 antigen in the periplasmic space, and the CsaC protein, an usher.

The CsaB and CsaE proteins cannot autonomously assemble to form the CS4 antigen. The CsaA and CsaC proteins are required for the assembly of the CS4 antigen. Thus, the combination of CsaB and CsaE will not result in formation of the fimbrial structure. Initial isolation of the CS4 operon included cloning a fragment including *csaA*, *csaB*, and truncated

csaC, on plasmid pKS-CSAII (Altboum et al., *Infect. Immun.* 71:1352-1360 (2003)). This plasmid encoding a complete CsaA and CsaB, but lacking a complete CsaC and CsaE, did not direct synthesis of CS4 fimbriae. Furthermore, Froehlich et al. (*Mol. Microbiol.* 12:387-401 (1994)) have demonstrated that the tip adhesin protein CooD of CS1, homologous to CsaE of CS4, was required for assembly of the fimbriae. CooB of CS1, which is homologous to CsaA of CS4, has been shown to have an essential role in fimbrial assembly (Scott et al., *Mol. Microbiol.* 6:293-300 (1992)).

We declare further that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 4/6/04

Eileen M. Barry, Ph.D.

Date: 4/6/04

Myron M. Levine, Ph.D.